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13. ABSTRACT ***(Maximum 200 Words)***

Transcription repression of E2F target genes has been shown to be critical for the maintenance of cellular quiescence and growth control. While it is clear that E2F-Rb and E2F-p130 complexes promote this transcriptional regulation, the mechanism of action of these complexes remains unclear. The work supported by this grant aims to elucidate the mechanisms of transcriptional repression through E2F-p130 complexes.
Previous results indicated that while repression through p130 involves recruitment of histone deacetylase, other mechanisms that are histone deacetylase independent are also involved. We have found that p130 can recruit the CtBP co-repressor protein through interaction with the CtIP protein. We have mapped the domain of CtBP that is important for its co-repressor function (PID domain) and data suggest that CtBP functions as a repressor in part by inactivating the histone acetyltransferase activity of the transcription co-activator, CBP.

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FOREWORD

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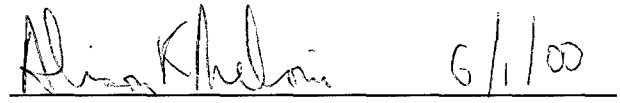

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Introduction:

The E2F transcription factor plays a critical role in directly inducing the expression of genes that are necessary for a cell cycle progression into S phase. During times of cellular quiescence when expression of S phase genes would be inappropriate, the retinoblastoma protein, Rb, is bound to E2F forming a complex that promotes active repression of E2F target genes. Mutations in the Rb protein or in the cyclin dependent kinase inhibitor p16 which regulates the activity of Rb, are common abnormalities in human breast cancer. As key regulators of E2F and the cell growth control pathway, a thorough understanding of the functions of these proteins and the pathways they regulate will undoubtedly benefit the diagnosis and treatment of breast cancer. Our research therefore aims to elucidate the mechanism by which Rb and family member protein, p130, convert E2F from a positive acting factor into a negative acting complex.

Body:

Objective 1: To identify the mechanism of p130 repression

We previously were able to show that p130 mediated transcription repression involves both histone deacetylase dependent and independent mechanisms. Using co-immunoprecipitation, we were able to show that Rb and p130 are capable of interacting with histone deacetylase. In addition, a mutation of Rb that was originally found in a human tumor, Rb C706F, that has been show to be ineffective as a transcriptional repressor [1], was incapable of interacting with histone deacetylase. This therefore defines a correlation between the ability of Rb to repress transcription and its ability to interact with histone deacetylase.

In addition to the examination of histone deacetylase as a potential mediator of transcriptional repression, we also examined proteins that we found in a yeast two hybrid assay to interact with the p130 protein. One protein, CtIP, was interesting in that it contains an LXCXE motif, a motif previously identified as being necessary for interaction with the pocket domain of Rb family proteins. As the pocket domain of Rb family proteins is the domain implicated in mediating transcriptional repression, we examined this protein further for its potential to mediate repression through p130. We found that CtIP like histone deacetylase can interact with both p130 and Rb in co-immunoprecipitation experiments. In addition, the same transcription repression defective mutant, Rb C706F, that was found not to interact with histone deacetylase is also incapable of interacting with CtIP [2]. This again correlates the ability of Rb and p130 to repress with their ability to interact with CtIP.

While we were investigating CtIP, it was published that CtIP interacts with a protein called CtBP and that CtBP has been shown in drosophila to act as a co-repressor [3-4]. We hypothesized that CtIP could also act as a co-repressor by recruiting CtBP. In fact a Gal4DNA binding domain fusion protein of CtIP was capable of repressing the SV40 promoter and mutation of the domain of CtIP that is important for its interaction with CtBP

decreases its ability to repress. We therefore were able to conclude that p130 is able to mediated repression both by the recruitment of histone deacetylase and by the recruitment of a CtIP/CtBP complex. That the histone deacetylase inhibitor, TSA, did not relieve repression of the SV40 promoter by CtIP or CtBP implies that this mechanism of repression is histone deacetylase independent.

We therefore decided to examine CtBP mediated repression. We found that CtBP was incapable of repressing the SV40 promoter in 293 cells, cells that contain E1A. As CtBP is known to bind to E1A through a PLDLS motif, we examined the importance of the PLDLS interacting domain (PID domain) of CtBP in transcription repression. Using a variety of mutants, we found that CtBP requires the PID domain to repress transcription.

Using the IBCP website we searched the Swissprot database for proteins containing a PLDLS like motif. One such set of proteins found to have a PMDLS motif, are the CBP/p300 transcription co-activators with intrinsic acetyltransferase activiy. As p300 and CBP act as transcription co-activators in part by their ability to acetylate histone, we examined if CtBP had an effect on the ability of CBP to acetylate histone. We found that in fact CtBP, but not a transcription repression defective mutant CtBP, can inactivate the acetylase activity of CBP. The action of inactivating a histone acetylase would in effect function similiarly to the recruitment of a histone deacetylase, that being the deacetylation of histone leading to transcription repression. It therefore seems that p130 represses transcription in complementary manners, recruitment of histone deacetylase and recruitment of the CtIP/CtBP complex which acts as a histone acetylase inhibitor.

The importance of the CtIP/CtBP complex in human cancer and particularly breast cancer has very recently come to light. Experiments of others show that many human tumor lines over express the CtIP mRNA [5]. In addition, the CtIP protein was recently shown to interact with the BRCA1 protein [6-7]. Interestingly, this interaction is abolished in several tumor-associated mutations of BRCA1 affecting the BRCT motifs indicating that in

addition to mediating the activity of Rb, CtIP could also be important for tumor suppression by BRCA1.

Objective 2: To identify a region of p130 responsible for p130 mediated repression.

While this objective remains viable and interesting to us, the success of objective 1 has opened new avenues of research that we have been eager to address. Unfortunately, time constraints have prevented us from obtaining any data about this objective.

Objective3: To investigate the possibility that E2F4 is mutated in human breast carcinomas.

Many breast cancer cell lines were obtained from ATCC. Many of these lines were analyzed for the expression of E2F4, however, none tested were found to lack expression or express mutant forms of the protein. As the MD fellow that guided this objective, Dr. Paul Kelly Marcom, has left the lab to take a position in the Oncology department at Duke Medical Center, no further examination of this objective has been undertaken.

Appendices

Key research accomplishments:

1) We have established that the CtBP has a histone deacetylase independent mechanism of transcription repression.

2) We found that the tumor virus protein, E1A has the ability to prevent CtBP from repressing transcription.

3) We defined a domain on CtBP (the PID domain) that is important for transcription repression.

4) We have shown that the CtBP protein is capable of inactivating the histone acetyl transferase activity of CBP.

List of Reportable Outcomes

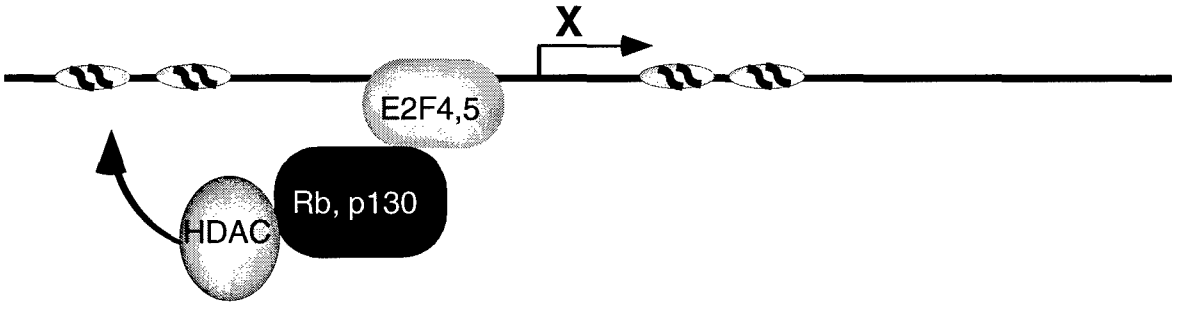
A manuscript " Complementary Mechanisms for E2F/Rb-Mediated transcriptional Repression-the CtBP Co-repressor Inhibits Histone Acetylase Activity of CBP" was submitted to Nature.

A poster "A mechanism for Rb/p130 mediated transcription repression involving the recruitment of the CtBP corepressor" was presented at the Keystone Symposia, Cancer, Cell Cycle and Therapeutics in January of 2000.

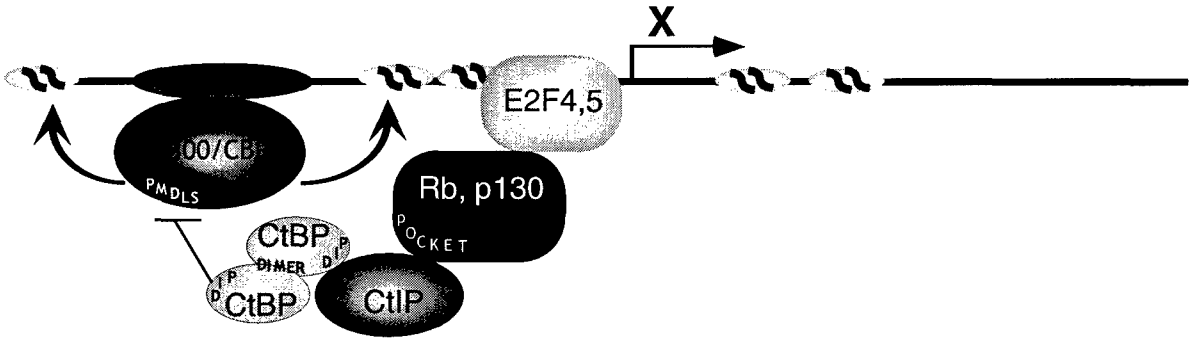
Conclusions

As described by the model on the following page, we propose that under certain conditions, E2F/Rb or E2F/p130 complexes can recruit histone deacetylase to modify chromatin structure at target promoters and prevent accessibility of transcription machinery resulting in repression. However, under other conditions, E2F/Rb or E2F/p130 complexes may bind to CtIP through a pocket domain/LXCXE motif interaction. CtIP would then interact with the PID domain of one molecule of the CtBP dimer, through a PLDLS motif, while the PID domain of the other molecule of CtBP remained free to associate with and inactivate the p300/CBP that exists as a co-activator for numerous transcription factors. The inactivation of CBP histone acetylase activity by CtBP therefore would prevent transcriptional activation by inhibiting acetylation of histones, pushing the overall state of the nucleosomes towards deacetylation and favoring transcription repression. This model represents a potential new mechanism for transcription repression. As nearly all promoters use CBP/p300 to coactivate transcription, inactivation of these coactivators could result in promoter silencing. It remains to be determined however if this mechanism alone is sufficient to repress transcription or if it contributes to a more complete repression together with other mechanisms that might recruit histone deacetylase.

Recruitment of Histone Deacetylase



Recruitment of a Histone Acetylase Inhibitory Complex



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ABSTRACT

Previous work has demonstrated the critical role for transcription repression in quiescent cells through the action of E2F-Rb or E2F-p130 complexes. Recent studies have shown that at least one mechanism for this repression involves the recruitment of histone deacetylase. Nevertheless, these studies also suggest that other events likely contribute to E2F/Rb-mediated repression. Using a yeast two hybrid screen to identify proteins that specifically interact with the Rb-related p130 protein, we demonstrate that p130, as well as Rb, interacts with a protein known as CtIP. This interaction is dependent on the p130 pocket domain, that is important for repression activity, as well as an LXCXE sequence within CtIP, a motif previously shown to mediate interactions of viral proteins with Rb. CtIP interacts with CtBP, a protein named for its ability to interact with the C terminal sequences of adenovirus E1A. Recent work has demonstrated that the Drosophila homologue of CtBP is a transcriptional co-repressor for Hairy, Knirps, and Snail. We now show that both CtIP and CtBP can efficiently repress transcription when recruited to a promoter by the Gal4 DNA binding domain, thereby identifying them as co-repressor proteins. Moreover, the full repression activity of CtIP requires a PLDLS domain that is also necessary for the interaction with CtBP. We propose that E2F-mediated repression involves at least two events, either the recruitment of a histone deacetylase or the recruitment of the CtIP/CtBP co-repressor complex. The elucidation of a mechanism for histone deacetylase-independent CtBP mediated repression is currently in progress.

134 **A mechanism for Rb/p130-mediated transcription repression involving the recruitment of the CtBP corepressor.**

Alison Meloni, Eric Smith, and Joseph Nevins, Department of Genetics, Duke University, Durham, NC, USA, 27710

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Complementary Mechanisms for E2F/Rb-Mediated Transcriptional Repression - the CtBP Co-Repressor Inhibits Histone Acetylase Activity of CBP

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Summary

E2F transcription activity includes a family of DNA binding activities that function both as transcriptional activating proteins as well as transcription repressors [1,2]. One group, including the E2F4 and E2F5 proteins, specifically associates with the Rb related p130 protein in quiescent cells [3], and functions to repress transcription of various genes encoding proteins important for cell growth. A series of recent reports has now provided evidence that one mechanism for this Rb-mediated repression involves an ability of Rb to recruit histone deacetylase to E2F-site containing promoters [4-6]. Nevertheless, several observations suggest that additional events contribute to E2F/Rb-mediated repression, including the fact that many genes subject to E2F/Rb-mediated repression are not de-repressed by treatment with the histone deacetylase inhibitor trichostatin A (TSA) [4]. Recently, we have shown that the recruitment of the CtBP co-repressor, a protein now recognized to play a widespread role in transcriptional repression, may represent one alternate mechanism for E2F/Rb mediated repression. We now show that the CtBP protein inhibits the histone acetylase activity intrinsic to the CBP transcription co-activator, dependent on a domain of CtBP that is required for transcription repression. Based on these results, it appears that E2F-mediated repression involves complementary events, either the recruitment of a histone deacetylase or the recruitment of a histone acetylase inhibitor.

Employing a yeast two hybrid screen to identify proteins that specifically interact with the Rb-related p130 protein, we identified a protein known as CtIP that interacts with the p130 pocket domain [7]. Similarly, others have shown that the CtIP/Rim protein interacts with Rb in vivo [8]. CtIP was previously described as a protein that interacts with CtBP, an adenovirus E1A-interacting protein [9,10]. Other work has shown that CtBP functions as a co-repressor in Drosophila [11-14] and various experiments have demonstrated a role for CtBP as a transcriptional repressor in mammalian cells [7,12-20]. Nevertheless, despite the clear evidence for a role of CtBP in transcriptional repression, a mechanistic basis for this repression, whether in the context of E2F/Rb or other transcription regulatory proteins, has not been elucidated. We now describe experiments that demonstrate that CtBP inhibits the histone acetylase activity of the p300/CBP class of transcriptional co-activators, dependent on CtBP sequences that are critical for transcription repression activity. It thus appears that E2F/Rb-mediated repression can involve complementary events that together lead to a loss of histone acetylation at target promoters.

Previous work has shown that the adenovirus E1A protein interacts with Rb and Rb family proteins as a mechanism for disrupting the growth suppressing activity of these proteins [21]. Interestingly, the CtBP protein was originally identified as a protein that bound to the C terminus of E1A [10], a domain distinct from that involved in binding to Rb family proteins. As such, we speculated that E1A might independently disrupt both the ability of Rb to interact with E2F as well as the ability of CtBP to function as a repressor. We have investigated this possibility using a Gal4-CtBP fusion protein that eliminates the contribution from p130 to CtBP repression. Although expression of the Gal4-CtBP fusion protein results in the substantial repression of the major late promoter (MLP) when assayed in C33A cells, a very different result is observed when the same assay is carried out in 293 cells. In this case, expression of Gal4-CtBP results in a slight activation of the MLP (Figure 1A). 293 cells express high

levels of E1A, suggesting the possibility that E1A might disrupt the ability of CtBP to mediate repression. To directly test this possibility, we again performed the Gal4-CtBP repression assay in C33A cells, this time adding varying amounts of E1A. As shown in Figure 1B, the addition of E1A together with Gal4-CtBP reversed the repression of the MLP by CtBP. As such, it would appear that E1A has the capacity to disrupt both forms of E2F/Rb-mediated transcription repression, either through the binding to Rb family proteins or through the interaction with CtBP.

Previous work has shown that the interaction of E1A with CtBP is dependent on a PLDLS sequence in the C terminal region of E1A [10,22]. In light of the finding that E1A can directly inhibit CtBP-mediated repression, it seemed possible that CtBP might require the domain through which it interacts with PLDLS motif containing proteins in order to mediate repression. Previous work has identified a centrally located dimerization domain within the 455 amino acid CtBP protein as well as a large and poorly defined domain necessary for interaction with PLDLS proteins that includes most of the N-terminus of the protein [19]. Given the possible role of the PLDLS-interaction in mediating repression, we generated a series of N terminal deletions to more precisely map the domain involved in PLDLS interaction and to identify the CtBP sequences important for transcriptional repression. Five such mutants were created lacking the first 10, 30, 40, 70 or 120 amino acids of the CtBP protein (Figure 2A). These mutants were first tested in a two-hybrid assay for their ability to interact with a PLDLS motif-containing protein, in this case the CtIP protein. While wild type CtBP and mutants lacking the first 10 or 30 amino acids retained the ability to interact with CtIP, mutants lacking the first 40, 70, or 120 amino acids all failed to interact with CtIP (Figure 2B). As a control, we find that all of the mutants can dimerize with wild type CtBP and are therefore functionally expressed in yeast.

We next assayed the ability of these CtBP mutants to repress transcription. The wild type and mutants of CtBP were made in the context of Gal4DBD fusions and transfected into C33A cells with the

SV40 promoter containing upstream Gal4DNA binding domain sites as a reporter. While the SV40 promoter was dramatically repressed by the wild type and mutants with the first 10 or 30 amino acids deleted, mutants containing deletions of the first 40, 70 or 120 amino acids were significantly impaired in their ability to repress the SV40 promoter (Figure 2C). Similar results were obtained using the MLP (data not shown). The data presented in Figure 3D shows that these proteins were all expressed at relatively equal amounts. These results thus define an N terminal domain that is essential for CtBP mediated transcription repression that also coincides with a PLDLS-interacting domain (PID).

Due to the importance of the PID domain in mediating repression by CtBP, we examined the possibility that CtBP might repress transcription by targeting a cellular factor that contains a PLDLS-like motif. By examining the group of proteins that are currently known to interact with CtBP through a PLDLS-like motif, it appears that most CtBP interacting proteins contain a PXDLS motif. We therefore chose to use the IBCP web site to identify instances of the pattern PXDLS in the Swissprot + SPT.EMBL protein database [23]. The database revealed a large number of proteins from all species, containing PXDLS motifs. We limited the number of candidate proteins by examining only human proteins with established function in transcription regulation (Table 1). Among this list were many transcription factors known to act as negative regulators of transcription, several of which are already known to bind CtBP. Interestingly, we also identified several transcription co-repressors, such as the proteins TGIF and methyl CpG binding protein, proteins found in complexes with histone deacetylase, and Tif1alpha, a nuclear receptor interacting repressor. As CtBP has been described to interact with HDAC and to exhibit sensitivity to TSA on certain promoters [17,24,25], it is possible that one mechanism of CtBP mediated transcription repression is through a HDAC dependent mechanism potentially through interactions with one or more of these proteins.

Several co-activators were also found to contain PXDLS motifs such as the 60 kd subunit of the mammalian Swi/Snf complex, a chromatin remodeling ATPase involved in transcription activation. An interesting set of co-activating PLDLS-motif containing proteins are the histone acetyltransferases, p300 and CBP. These two proteins contain a PMDLS motif in the bromodomain, located just upstream from the histone acetylase catalytic domain. The finding that CBP and p300 may contain a CtBP interacting motif was intriguing given the recent data that the E1A, E6, and Twist proteins can inactivate the acetyltransferase activity of p300/CBP [26-28]. We have therefore sought to determine if CtBP may also have the ability to inactivate p300/CBP.

Given the role of the intrinsic histone acetylase activity in the function of CBP as a co-activator, we measured the effect of CtBP on the histone acetylase activity of CBP. As shown in Figure 3A, incubation of CBP with CtBP followed by addition of histone, and ^{3}H acetyl CoA resulted in a substantial inhibition of acetylase activity. As a control, boiled CtBP had no effect on CBP acetylase activity (data not shown). We further examined the dose effect of the CtBP inhibition of CBP. Figure 3B shows that a 20 minute preincubation of CBP with increasing doses of CtBP produced an increasingly dramatic inactivation of CBP relative to the BSA control. These data indicate that the CtBP corepressor can significantly inactivate the histone acetylase activity of CBP in vitro.

To determine whether the inactivation of CBP histone acetylase activity reflects the CtBP-mediated transcription repression, we assayed the ability of the various CtBP mutants for their ability to affect acetylase activity. We preincubated BSA, CtBP, CtBP$^{\Delta 1\text{-}10}$ or CtBP$^{\Delta 1\text{-}120}$ with CBP for 20 minutes followed by the addition of histone and ^{3}H-acetyl CoA. While CtBP and CtBP$^{\Delta 1\text{-}10}$ dramatically inhibited the acetylation of histones by CBP relative to the BSA control, CtBP$^{\Delta 1\text{-}120}$ was severely impaired in its ability to inactivate CBP (Figure 3C). The same result was obtained in multiple experiments and with different preparations of CtBP and the mutant proteins. These results correlate the

ability of CtBP to repress transcription with its ability to inactivate CBP, indicating that one mechanism of CtBP mediated transcription repression is through the inactivation of acetylase of activity of CBP. While CtBP clearly inactivates CBP acetylase activity, we have been unable to demonstrate a stable interaction between CtBP and CBP by standard co-immunoprecipitation assays suggesting that such an interaction may be weak or transient.

The role of localized histone acetylation, as a mechanism for control of transcription activity has now been shown in multiple instances. This includes both the recruitment of histone acetylases as a mechanism for transcription activation, as well as the recruitment of histone deacetylases as a mechanism of transcriptional repression. Since CBP-like co-activators with intrinsic HAT activity are likely to be a common component of many transcriptional activation events, the role of a histone acetylase inhibitor is logical and could be of wide-spread importance. In the case of E2F/Rb-mediated repression, the inhibition of acetylase activy of p300/CBP suggests a complementary event for E2F/Rb-mediated repression. In one case, the recruitment of histone deacetylase can lead to transcriptional repression through the localized alteration of chromatin structure [4-6]. This action alone, however, might be expected to be less than complete given the expected continued histone acetylation mediated by promoter-bound acetylase. Our observation that E2F/Rb can also recruit a histone acetylase inhibitor would then allow a complete inhibition of histone acetylation. Taken together, these data are consistent with the model diagrammed in Figure 4. We propose that under certain conditions, E2F/Rb or E2F/p130 complexes can recruit histone deacetylase or CtBP to modify chromatin structure at target promoters, blocking the accessibility of the transcription machinery, and thus resulting in repression. Since a large number of promoters likely use CBP/p300 to coactivate transcription, inactivation of these coactivators could result in promoter silencing. The complementary nature

of this model is intriguing given that most promoters of E2F target genes contain more than one E2F site. Perhaps the two sites work together to mediate repression, one recruiting HDAC and another recruiting the HAT inactivating protein, CtBP. In this manner, the presence of both HDAC and a HAT inactivating protein on the same promoter would insure local chromatin structure containing hypoacetylated nucleosomes and thus transcription repression. Yet another characteristic of E2F sites is that they often exist very close to the transcription start site. This is interesting in view of the finding that several Drosophila short range repressors, repressors that function at <100 bp away from the transcription initiation site, appear to function through the recruitment of CtBP [13].

The ability of the adenovirus E1A protein to alter cell proliferation is tightly linked to the ability of E1A to interact with a complex series of proteins each of which is involved in transcription control. This includes the binding of E1A to the Rb family proteins, disrupting the interaction with E2F. This prevents the ability of Rb to suppress activation function of E2F1-3 and also disrupts the repressor complexes involving either HDAC or CtBP. Although the interaction of E1A with Rb family proteins would also lead to the disruption of the CtBP-containing complex, the ability of E1A to directly bind to CtBP provides a second mechanism by which E1A might interfere with E2F-dependent transcription repression. In addition, two recent reports describe yet another mechanism for E1A action that involves a direct inhibition of histone acetyl transferase activity [26,28]. In addition to its ability to disrupt complexes involving the p300 protein, these two reports demonstrate that the direct interaction of E1A with either p300/CBP or PCAF leads to an inhibition of histone acetylase activity. As such, it appears that the E1A protein has evolved a series of distinct activities to affect transcription through an alteration of chromatin structure.

Experimental Procedures

Cell culture

C33A cells and 293 cells were grown in DMEM with 10% fetal bovine serum. Sf9 cells were grown in suspension in HyQ SFX-insect without serum.

Plasmids and reagents

The SV40 promoter with upstream Gal4 sites (pSVECG) was a kind gift from D. Dean [29]. The MLP with Gal4 sites was a kind gift from D. Dean and R. Eisenman. The Gal4p130, Myc-CtIP, Gal4BD CtBP, and Gal4AD CtIP constructs were made as described [7].

Gal4BD CtBP was made by PCR using 5'-AAAAGAATTCATGGGCAGCTCGCACTT GCT-3' and 5'-AAAATCTAGACTACAACTGGTCACTGGCGT-3' as primers using the CtBP clone (kind gift from G. Chinnadurai) as a template. Gal4BD CtBP$^{\Delta 1\text{-}10}$ was created by PCR using 5'-AAAAGAATTCATGCTGCCGCTTGGCGTCCGACCT-3' and 5'-AAA ATCTAGACTACAACTGGTCACTGGCGT-3' as primers. Gal4BD CtBP$^{\Delta 1\text{-}30}$ was created by PCR using 5'-AAAAGAATTCATGCCCCTGGTGGCATTGCTGGA-3' and 5'AAAATCT AGACTACAACTGGTCACTGGCGT-3' as primers. Gal4BD CtBP$^{\Delta 1\text{-}40}$ was made by PCR using 5'-AAAAGAATTCATGCCCATCCTGAAGGACGT-3' and 5'-AAAATCTAGACT ACAACTGGTCACTGGCGT-3' as primers. Gal4BD CtBP$^{\Delta 1\text{-}70}$ was made by PCR using 5'-A AAAAAGAATTCATGGCTGTGGGGGCCCTGATYGTA-3' and 5'-AAAATCTAGACT ACAACTGGTCACTGGCGT-3' as primers. Gal4BD CtBP$^{\Delta 1\text{-}120}$ was made by PCR using 5'-AAAAGAATTCATGGCGTCTGTGGAGGAGACGGCC-3' and 5'-AAAATCTAGACT ACAACTGGTCACTGGCGT-3' as primers.

The CtBP baculoviruses were made by cloning CtBP into the Eco RI – Xba I sites of the pFASTBAC Hta vector. Virus was made according to the BAC-TO-BAC Baculovirus Expression Systems protocol (Gibco Life Technologies) The Flag-CBP virus was made as described [30].

Repression assays

C33A cells were transiently transfected by the calcium phosphate method with the described DNA plasmids. A β-gal expressing plasmid (1 μg) was cotransfected as a control for transfection efficiency. After 15 hours, cells are washed 2X with PBS and allowed to recover in DMEM with 10% serum. 40 hours post transfection cells are harvested and CAT assays are performed as described in Maniatis with the following modifications: Extracts are not heat inactivated. CAT assay reaction mixture includes 75 μl extract, 75 μl 1M Tris.Cl (7.8) 1 μl ^{14}C-labled chloramphenicol (1 mCi/ml), and 30 μl of acetyl coenzyme A (3.5 mg/ml in H2O). Reactions are incubated at 37° for 3.5 hours. Raw CAT values are normalized relative to the vector alone control β-gal values. β-gal was measured by adding 10 μl of the extract prepared for the CAT assays to 590 μl of 0.1mg/ml CPRG in Z buffer. Absorbance was measured at 570. In all instances of comparison, westerns were performed to determine that equal protein levels are expressed.

Yeast two-hybrid assays

The yeast two hybrid is performed as recommended in the CLONTECH protocol.

Baculovirus expressed proteins

Purified bacuovirus expressed proteins were made by infecting 50 mls of 2 x 10^6 cells/ml SF9

cells with a given virus. 48 hours post infection, cells were harvested and lysed in 50mM Tris-HCl, 1% NP40, 10mM BME, 1mM PMSF, 2 μg/ml Pepstatin, and 2 μg/ml Aprotinin. Extracts were then incubated for 30 minutes at 4 degrees with 1ml of a 50% slurry of Nickel beads. Beads were loaded onto a column and washed. Protein was eluted in 20mM Tris-HCL, 100mM KCl, 100mM imidizole, 10% glycerol, and 10mM BME. 0.5 ml fractions were collected and peak fractions 2,3 and 4 were pooled.

Histone acetylase assays

Approximately 150 ng of baculovirus purified Flag-CBP was preincubated with 15 μg of BSA, baculovirus purified CtBP, or CtBP mutants in a 15 μl volume with buffer containing 50mM Tris-HCl, 10% glycerol, 1mM DTT, 1mM PMSF, 0.1mM EDTA, 75mM NaCl, and 10mM Butyric Acid for 20 minutes at 30 degrees. After preincubation, 25 μg histones and 125 μCi of ^{3}H-acetyl CoA were added and the volume raised to 30 μl keeping the buffering conditions constant. The reaction was allowed to proceed at 30^{o} C for 20 more minutes after which the reactions are immediately boiled and run on a 15% acrylamide gel. The gel is fixed, amplified, dried and exposed to film. After exposure, the gel is rehydrated and stained with Coomassie blue.

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Table 1. Identification of PXDLS-containing proteins

Protein	Sequence
DNA-binding transcription factors	
Two-handed zinc finger protein ZEB	PLDLS
SMAD6	PLDLS
Transcription factor 8 (Delta EF1)	PLDLS
AP-2REP transcription factor	PVDLS
Negative-regulator of IL-2	PLDLS
cAMP response element binding protein CRE-BP1 (ATF2)	PLDLS
Zinc finger protein FOG-2	PIDLS
DNA-binding protein IKAROS	PEDLS
Zinc finger protein HRX	PSDLS
Ras-responsive element binding protein 1 RREB-1	PIDLS
T-brain-1 protein TBR-1	PKDLS
Retinoblastoma protein interacting zinc finger protein RIZ	PLDLS
Transcription co-repressors	
CtBP interacting protein CtIP	PLDLS
Retinoblastoma interacting protein RIM	PLDLS
5'-TG-3' interacting factor TGIF	PLDLS
Methyl-CpG binding protein 2 MECP-2	PQDLS
Receptor interacting protein 140	PIDLS
Transcription intermediary factor 1-alpha	PMDLS
Transcription co-activators	
E1A-associated protein p300	PMDLS
CREB-binding protein CBP	PMDLS
RING3	PMDLS
Thyroid hormone receptor co-activating protein	PMDLS
SWI/SNF complex 60 KD subunit	PGDLS

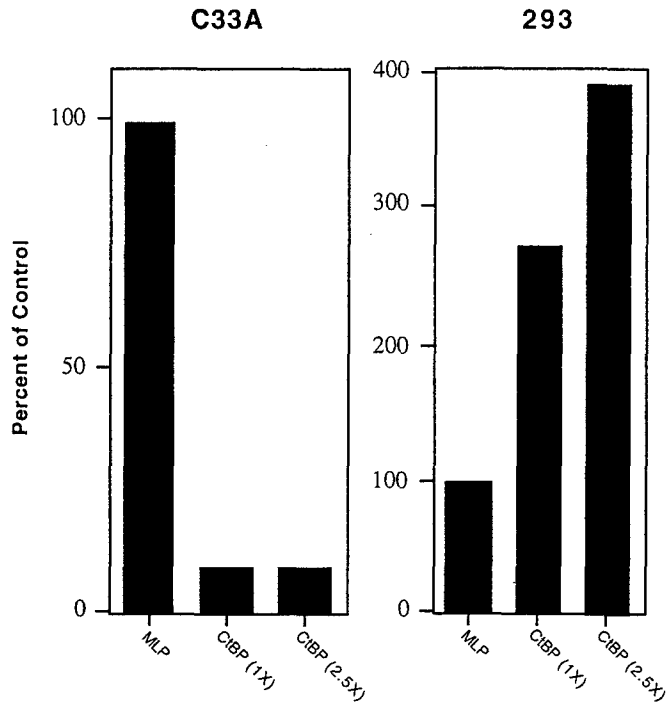
The SWISSPROT + SPTrEMBL protein database was searched for instances of the pattern PXDLS using the IBCP web site. This list was then limited to human proteins known to play a role in transcription. These proteins were then grouped in 3 general categories: DNA binding transcription factors, transcription co-repressors, or transcription co-activators.

Figure 1. E1A disrupts CtBP-mediated transcription repression.

A. C33A or 293 cells were transiently transfected with 1μg of CMV β-gal, 0.5μg MLP reporter, and 2μg or 5μg of Gal4-CtBP. Cells were harvested 40 hours posttransfection, and CAT activity was assayed. β-gal values were used to normalize for transfection efficiency. The experiment was repeated at least three times and the results of a typical experiment are shown.

B. C33A cells were transiently transfected as in panbel A except 1 μg or 5 μg of E1A plasmid was added where indicated. Percent control for MLP represents expression from the ML promoter vector expressed with and without E1A and set to 100%. The experiment was repeated at least three times and the results of a typical experiment are shown.

A


C33A
293
Percent of Control
100
50
0
400
300
200
100
0
MLP
CtBP (1X)
CtBP (2.5X)
MLP
CtBP (1X)
CtBP (2.5X)

B

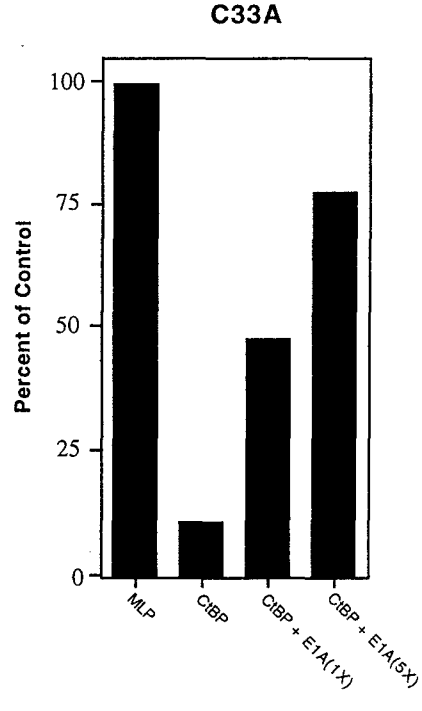

C33A
Percent of Control
100
75
50
25
0
MLP
CtBP
CtBP + E1A(1X)
CtBP + E1A(5X)

Figure 2. Identification of a domain within CtBP essential for PLDLS interaction and transcription repression

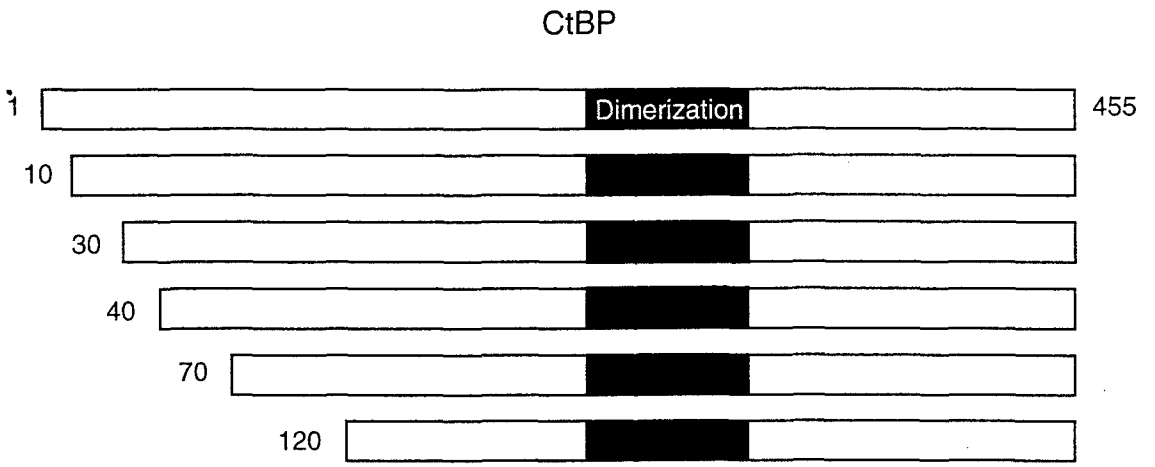
A. A schematic representation of CtBP along with the N-terminal deletion mutants.

B. HF7C yeast were transformed with plasmid encoding Gal4DBD-CtBP or each of the mutants along with Gal4AD-CtIP, Gal4AD-CtBP or Gal4AD. Yeast were then plated on media lacking Trp, Leu, and His that is selective for protein/protein interactions. Yeast that grew on selective media were then assayed for the presence of β-gal. An interaction was scored positive if yeast could both grow on selective media and express β-gal.

C. The Gal4-CtBP wild type and mutants were cloned into mammalian expression vectors and transfected into C33A cells with β-gal, and pSVECG (SV40) reporter plasmid. Cells were harvested 40 hours posttransfection, and CAT activity was assayed. β-gal values were used to normalize for transfection efficiency. Results of a typical experiment are shown.

D. Western blot, using Gal4DNA binding domain antibody, of C33A transfected Gal4-CtBP and mutants. β-gal was used as a loading control for transfection efficiency.

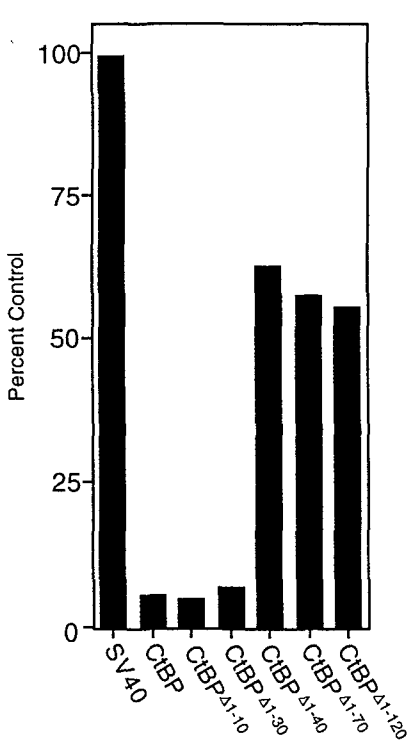
A



B

	CtBP	CtBP Δ1-10	CtBP Δ1-30	CtBP Δ1-40	CtBP Δ1-70	CtBP Δ1-120
Gal4AD CtIP	+	+	+	–	–	–
Gal4AD CtBP	+	+	+	+	+	+
Gal4AD	–	–	–	–	–	–

C



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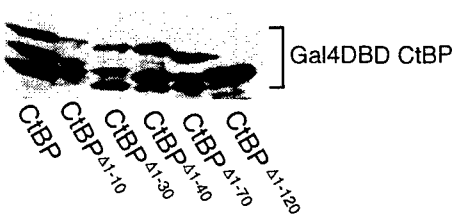


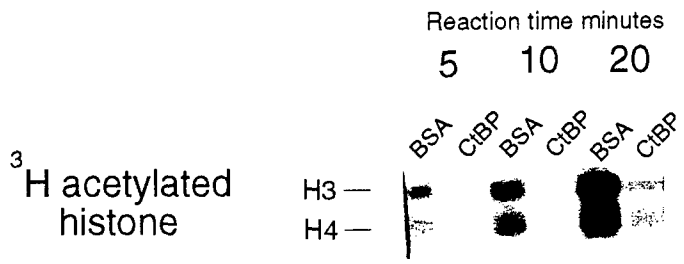
Figure 3. CtBP inhibits histone acetylase activity of CBP

A. Baculovirus-expressed Flag-CBP was pre-incubated for 20 min with baculovirus-expressed 6His-CtBP or an equal amount of BSA as a control. ^{3}H-acetyl coenzyme A and histone were then added to the reaction which was allowed to proceed for the indicated time. Products were analyzed in a 15% acrylamide gel.

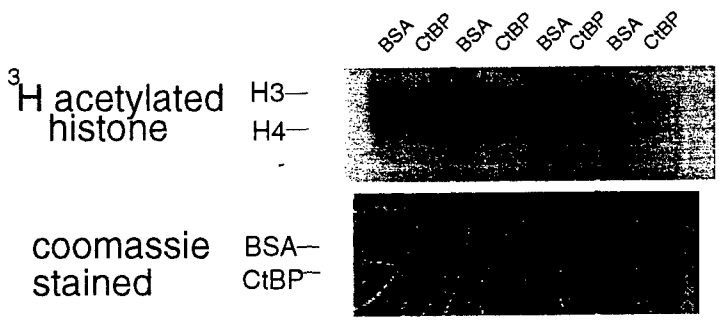
B. Baculovirus-expressed Flag-CBP was pre-incubated for 20 min with increasing amounts of baculovirus-expressed 6His-CtBP or BSA. ^{3}H-acetyl coenzyme A and histone were then added to the reaction which was allowed to proceed for 20 min.

C. Baculovirus-expressed Flag-CBP was pre-incubated for 20 min with baculovirus-expressed 6His-CtBP, CtBP$^{\Delta 1\text{-}10}$, CtBP$^{\Delta 1\text{-}120}$ or an equal amount of BSA as a control. ^{3}H-acetyl coenzyme A and histone were then added to the reaction which was allowed to proceed for 20 min. This experiment is a representative of 6 separate assays with two different preparations of purified proteins.

A


Reaction time minutes
5
10
20
BSA
CtBP
BSA
CtBP
BSA
CtBP
^{3}H acetylated histone
H3
H4

B


BSA
CtBP
BSA
CtBP
BSA
CtBP
BSA
CtBP
^{3}H acetylated histone
H3
H4
coomassie stained
BSA
CtBP

C

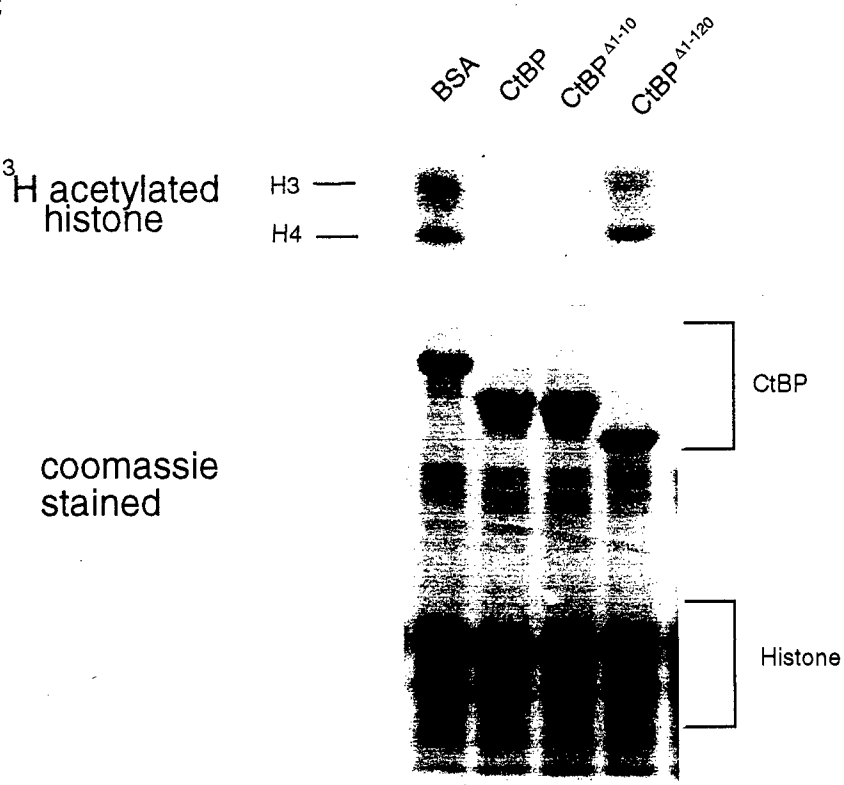
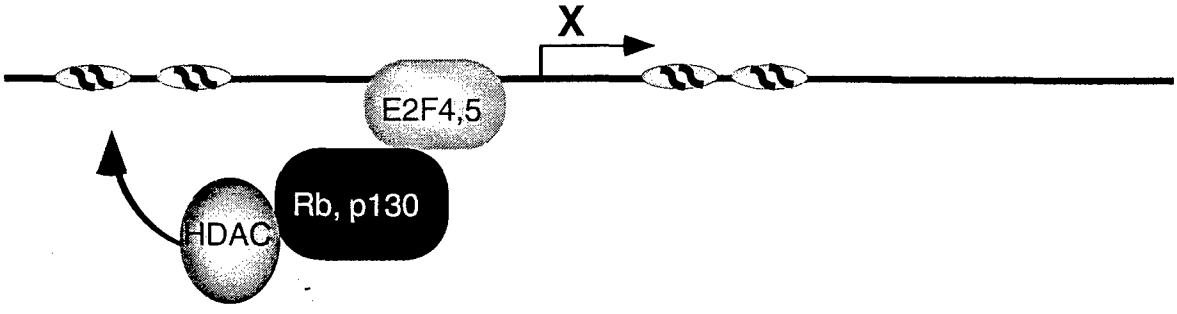

BSA
CtBP
CtBP$^{\Delta 1-10}$
CtBP$^{\Delta 1-120}$
^{3}H acetylated histone
H3
H4
coomassie stained
CtBP
Histone

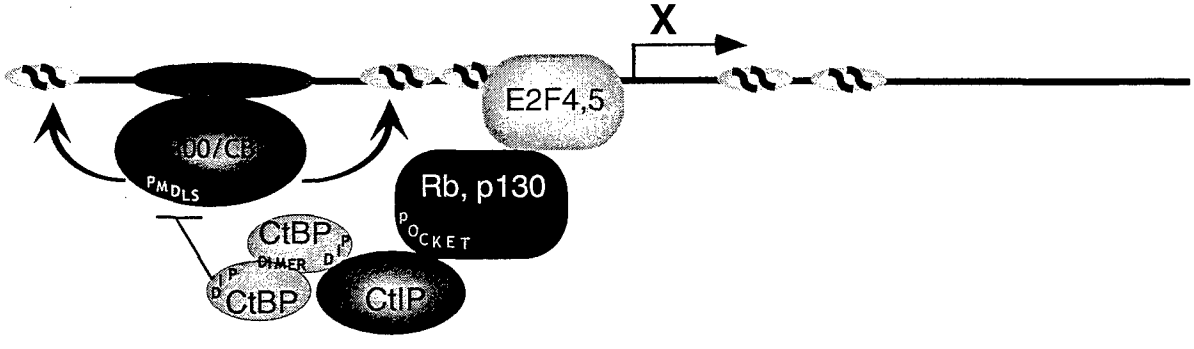
Figure 4. Complementary mechanisms for E2F/Rb mediated transcription repression

E2F target promoters can be repressed in two fashions. Histone deacetylase is recruited to promoters that contain E2F/Rb or E2F/p130 complexes through an interaction with Rb or p130. Histone deacetylase then modifies the histones proximal to the promoter causing transcriptional silencing. Rb and p130 recruit CtIP/CtBP to E2F complexes. CtIP bridges the interaction between CtBP and the E2F/Rb complex. CtBP, acting as a dimer, then functions by inhibiting p300/CBP bound to the promoter through basal transcription machinery or other transcription factors.

Recruitment of Histone Deacetylase



Recruitment of a Histone Acetylase Inhibitory Complex



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